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This project aims to understand the biologic functions of the small GTPase Rap-1, the mechanism by which overexpression or overactivation of Rap-1 can antagonize the promitogenic actions of Ras, and to determine whether strategies can be devised to recruit this antioncogenic function of Rap-1 to treat breast cancer.

Initial studies using a high affinity polyclonal antibody specific for Rap-1 indicate that Rap-1 is expressed in many cell lines, including the MCF-7 breast cancer line. Preliminary studies in Rat-1 cells indicate that endogenous Rap-1 may associate with the Raf-1 protooncogene in situ in a regulated fashion; Raf-1 is a critical mitogenic effector of the Ras protooncogene. The effect of Rap-1 association on the activation of the Raf-1 kinase and the downstream MAP kinase cascade is not yet known.

Expression cloning of Rap-1 interacting proteins has yielded a large number of Raf-1 sequences, many isoforms of guanyl nucleotide exchange proteins for other small GTPases, and a variety of proteins of unknown function; several of the latter are multiply represented, and contain interesting regulatory domains, but lack unmistakable catalytic domains. The role of these polypeptides in Rap-1's biologic program and potential antioncogenic action remains to be uncovered.

Future studies will define more fully the interactions of Rap and Raf in situ, the regulation of this coupling and the significance to Rafs mitogenic signalling. The role of the other candidate Rap-1 partners in Rap-1s physiologic and antioncogenic actions will be determined.

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Joseph Aurnch 12/1/95

PI - Signature Date

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Introduction

The Ras protooncogenes are a family of small GTPases that serve a central and indispensable role in the mitogenic action of receptor tyrosine kinases, and in mutant form are among the most commonly encountered oncogenes in human cancer. In breast cancer, Ras mutations are rare but Ras overexpression is frequent. Moreover, Ras function is absolutely required for the growth-promoting action of the mutant, constituitively active ErbB2 tyrosine kinase oncogene that occurs in two thirds of human breast cancer (1,2). Consequently, antagonism of Ras function is an attractive target for anti-neoplastic interventions.

The Rap-1 polypeptides are another subfamily of small GTPases, approximately 50% identical in sequence to the Ras polypeptides (3). The Rap polypeptides are not mitogenic; when overexpressed, Rap-1 has the ability to cause reversion of Ras induced transformation in some cell backgrounds. Thus Rap-1 is a naturally occurring potential antioncogene, although its normal role in cell regulation is not yet clear. The goal of the present work is to gain a clearer understanding of the normal function of Rap-1; to define the conditions that enable Rap-1 to function as an antioncogene in breast carcinoma cell lines, to define the biochemical mechanism by which overexpressed Rap-1 acts as a Ras antagonist.

Body: 8/94-8/95

During this preceding year, efforts have focused on two overall goals.

1. We have sought to develop the reagents necessary to examine Rap-1 function in situ, particularly a specific high affinity antiserum to the Rap-1 polypeptides. This will enable isolation of the Rap-1 polypeptides by immunoprecipitation, and detection by immunoblot.

We synthesized (through Research Genetics, Inc) a peptide corresponding to residues 121-137 of the Rap-1 protein coupled to the carrier MAP and immunized New Zealand White Rabbits. After several boosts, immunoblot of extracts(50 microgram protein per lane) prepared from a variety of cell lines, using antisera at >1:000 dilution, revealed a single immunoreactive band at about 25KDa, consistent with Rap-1 (fig. 1A). Interestingly, the relative abundance of Rap-1 in extracts from the MCF-7 breast cancer cell line was considerably greater than in Hela, hepatoma or fibroblast cell lines.

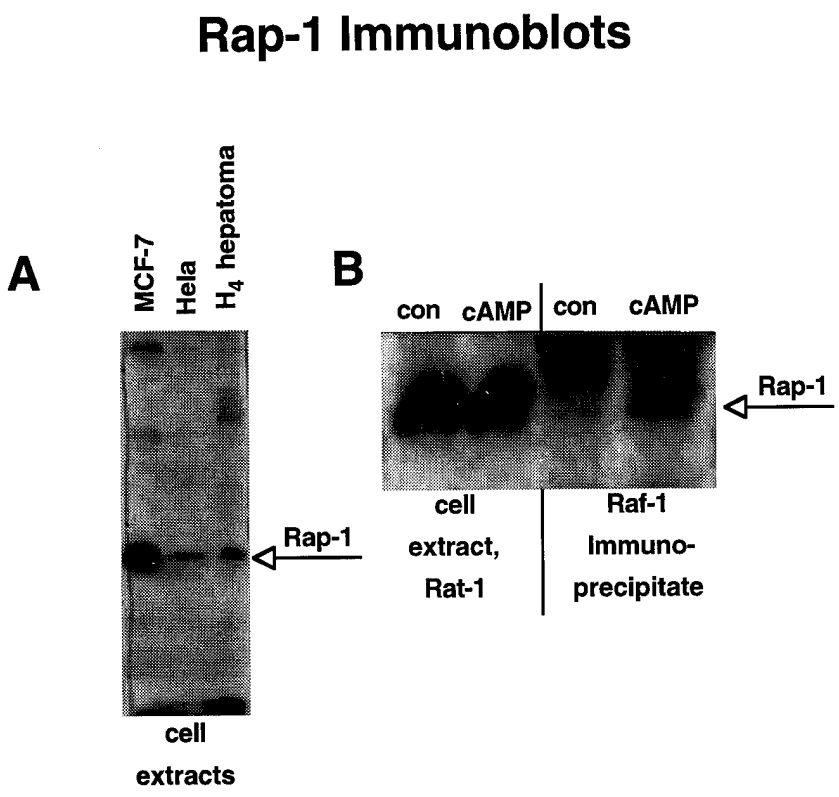

Figure 1A Figure 1B

Using this Rap-1 antibody, we have initiated studies of the regulation of Rap-1, and its possible association with elements in the Ras-Raf

cascade. Our earlier studies (4) indicated that the aminoterminal segment of the cRaf-1 kinase binds to the effector loop of Rap-1 in a yeast two-hybrid expression system with an affinity comparable to that exhibited by Ras. We therefore attempted to determine whether an association between Rap-1 and Raf was detectable in mammalian cells, in situ. The Raf-1 polypeptide was immunoprecipitated from extracts of Rat-1 cells, and the precipitate was subjected to anti Rap-1 immunoblot after SDS PAGE (fig. 1B). No evidence of a Rap-Raf complex was detected in resting cells, however after a 2 hour treatment of the Rat-1 cells with 0.5 mM 8BrcAMP prior to harvest, a 25KDa polypeptide immunoreactive with anti Rap-1 antiserum was observed to coimmunoprecipitate with cRaf-1. Inasmuch as cAMP has recently been reported to increase the fraction of Rap-1 in the GTP bound state (5), our finding suggests that Rap-1/GTP can bind to Raf in situ and this association may be regulated by cyclic AMP. In further experiments we determined that detection of this complex appeared to be dependent on the state of cell growth and confluence, as well as the time after cAMP activation. Inasmuch as the detection of these elements is limited by the low abundance of the endogenous components, it is our intention to further characterize these interactions utilizing transient overexpression, before returning to studies of endogenous components in tumor cell lines.

2. A second direction of the work has been an effort to identify potential effector molecules of the activated Rap polypeptide, utilizing the yeast interaction expression cloning method known as the "two hybrid" technique. A Rap-1B cDNA was inserted downstream of sequences encoding a Gal-1 DNA binding domain and a murine T cell cDNA library, fused inframe with the Gal-1 transcriptional activation domain (II) was screened for sequences that enabled growth on His^- (minus) media, and confer expression of a beta-galactosidase reporter. In a screen of over $5x10^6$ transformants, 45 cDNAs were recovered that survived all screening tests. Sequence analysis of these revealed that the 10 cDNAs encoded members of the Raf kinase family (nine A Raf, one c-Raf 1).

This high frequency of Raf sequences is highly supportive of the likelihood that Raf is one of the physiologic targets of Rap action and this area is already under study as described above.

Another 14 cDNAs encoded a variety of related polypeptides related(60-90% identity) to each other in sequence, which are homologous to the guanyl nucleotide exchange proteins for the small GTPase, Ral. Altogether, six different sets of such cDNAs was recovered, including two that had previously been reported as in vitro binding partners and potential effectors of the Ras polypeptides. Some of these GDS homologues are only 60-70% identical in aminoacid sequence to the Ral GDS a and b, suggesting that Rap interacts with and controls the regulatory proteins for an array of other small GTPases.

The remaining 21 isolates include 4 sets of multiply recovered cDNAs, and 10 individual isolates. None of these sequences, save one, have yet to be isolated from mammalian sources, and while some exhibit interesting domains, e.g. such as a pleckstrin homology domain, none possess an identifiable catalytic domain.

Conclusions:

The work carried out over the previous year has identified several potential effectors of the Rap GTPase. The Raf protooncogenes appear to interact with Rap in situ in a regulated fashion, and a continued analysis of the regulation of Rap-Raf binding, and the effect of Rap on the activation of the Raf kinase may provide important insights into the mechanism for the recruitment of the antioncogenic activity of Rap

The identification of additional Rap partners enables a more comprehensive view of Rap action. A central question is whether any of the potential Rap partners exhibit preferential binding to Rap over Ras; which of these candidates bind to the Rap effector domain; whether any of these new partners exhibit antimitogenic or

promitogenic activity on their own, especially if provided with the C-terminal targetting sequences of Ras or Rap; whether these biologic functions are regulated by c-AMP; whether any of these partners are expressed in breast cancer cell lines.

The availability of a specific anti-Rap-1 antiserum enables the study of the regulation of Rap-1 function (Task 4) to proceed in the same set of experiments as the characterization of the Ras/MAP kinase pathway in breast cancer cell lines(Task 2), and avoids duplication of these programs of experiments.

References

1. Muller, W. J., Sinn, E., Pattengale, P.K., Wallace, R. and Leder, P. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell***38**:627-637, 1984.

2. Kraus, M.H., Fedi, P., Starks, V., Murano, R. and Aaronson, S. A. Demonstration of ligand independent signalling by erb b-3 tyrosine kinase and its constituitive activation in human breast tumor cells. *Proc. Natl. Acad. Sci. USA***90**:2900-2904, 1993.

3. Noda, M. Mechanisms of reversion. *FASEB***7**:834-840, 1993.

4. Zhang, X-f., Settleman, J., Kyriakis, J.M., Takeuchi-Suzuki, E., Elledge, S.J., Marshall, M.S., Bruder, J. T., Rapp, U.R., and Avruch, J. Normal and oncogenic $p21^{ras}$ proteins bind toothe amino-terminal regulatory domain of c-Raf-1. *Nature* **364**:308-313, 1993.

5. Durfee, T., et.al. *Genes Develop.***7**:555-569, 1993.

6. Albright, C.F., Giddings, B.W., Liu, J., Vito, M., and Weinberg, R.A. Characterization of a quanine nucleotide dissociation stimulator for a *ras*-related GTPase. *EMBOI* :339-347,1993.

7. Kikuchi, A., Demo, S.D., Ye, Z-H,, and Williams, L.T., ralGDS family members interact with the effector loop of *ras*p21. *Mol. Cell Biol.***14**:7483-7491, 1994.